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Effect of $P2X_7$ receptor knockout on exocrine secretion of pancreas, salivary glands and lacrimal glands

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The purinergic $P2X_7$ receptors are expressed in different cell types where they have varied functions, including regulation of cell survival. The $P2X_7$ receptors are also expressed in exocrine glands, but their integrated role in secretion is unclear. The aim of our study was to determine whether the $P2X_7$ receptors affect fluid secretion in pancreas, salivary glands and tear glands. We monitored gland secretions in *in vivo* preparations of wild-type and $P2X_7^{-/-}$ (Pfizer) mice stimulated with pilocarpine. In cell preparations from pancreas, parotid and lacrimal glands we measured ATP release and intracellular Ca^{2+} activity using Fura-2. The data showed that pancreatic secretion and salivary secretions were reduced in $P2X_7^{-/-}$ mice, and in contrast, tear secretion was increased in $P2X_7^{-/-}$ mice. The secretory phenotype was also dependent on the sex of the animal, such that males were more dependent on the $P2X_7$ receptor expression. ATP release in all cell preparations could be elicited by carbachol and other agonists, and this was independent of the $P2X_7$ receptor expression. ATP and carbachol increased intracellular Ca^{2+} activity, but responses depended on the gland type, presence of the $P2X_7$ receptor and the sex of the animal. Together, these results demonstrate that cholinergic stimulation leads to release of ATP that can via $P2X_7$ receptors up-regulate pancreatic and salivary secretion but down-regulate tear secretion. Our data also indicate that there is an interaction between purinergic and cholinergic receptor signalling and that function of the $P2X_7$ receptor is suppressed in females. We conclude that the $P2X_7$ receptors are important in short-term physiological regulation of exocrine gland secretion.

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Introduction

Mammalian exocrine glands, such as pancreas, salivary and lacrimal glands, have different functions, but overall similar morphology. They comprise acini/endpieces and series of more or less elaborate ducts. Generally, acini/endpieces secrete fluid rich in NaCl and proteins and ducts modify this primary secretion by processes of secretion and/or reabsorption. Different transport processes in these glands lead to secretion of isotonic bicarbonate-rich secretion of pancreas, hypotonic salivary secretion and isotonic tear secretion. In recent years it became apparent that purinergic signalling may be an important regulator of exocrine glands (Novak, 2003). Exocrine cells can release ATP in response to neuronal or hormonal stimuli, as shown in a few studies (Sørensen & Novak, 2001; Yegutkin *et al.* 2006; Ishibashi *et al.* 2008). There are a number of purinergic receptors expressed on epithelial cells of acini and ducts, especially well studied in pancreas and salivary glands (Ishibashi *et al.* 2008; Novak, 2008; Nakamoto *et al.* 2009).

P2 purinergic receptors include receptors belonging to the two families: G-protein coupled P2Y receptors and ligand-gated ion channel P2X receptors. One of the most intriguing receptors is the $P2X_7$ receptor. Originally it was found on cells of hematopoietic lineage, but later it was localized to the nervous system (astrocytes, microglia, neurons), as well as to bone, epithelia, other tissues (Khakh & North, 2006; Sperlagh *et al.* 2006; Novak, 2008), and even cell organelles (Amstrup & Novak, 2003; Fountain *et al.* 2007). This receptor, often studied in cell preparations and stimulated by relatively high concentration of ATP, causes release of cytokines, activates caspase-1 and forms or triggers formation of lytic pores in many cells (North, 2002; Donnelly-Roberts & Jarvis, 2007). However, in some cell types and/or depending on the extent of stimulation, it can stimulate proliferation and may enhance the efficiency of mitochondrial metabolism (Donnelly-Roberts & Jarvis,

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2007; Di Virgilio *et al.* 2009). Overall, the $P2X_7$ receptor seems to have many functions in short- and long-term processes including inflammation, neuropathic pain, bone remodelling and neuro-modulation (North, 2002; Burnstock, 2008; Romagnoli *et al.* 2008). Some variability of receptor effects may depend on genetic signatures. There are 10 splice variants of the receptor found in various tissues, but only some are functional (Cheewatrakoolpong *et al.* 2005; Nicke *et al.* 2009); and the receptor is also a site of many single nucleotide polymorphisms (Ohlendorff *et al.* 2007; Fuller *et al.* 2009). Interestingly, a recent study revealed a new splice variant, $P2X_{7(k)}$, which has a novel alternative exon 1 and translational start, and therefore escaped gene inactivation in the Glaxo $P2X_7^{-/-}$ mouse (Nicke *et al.* 2009). In contrast, the Pfizer $P2X_7^{-/-}$ mouse does not express the $P2X_7$ receptors as far as is known.

What then is the function of the $P2X_7$ receptor in exocrine glands? On a cellular level, it was recognized years ago that ATP stimulated cation currents and fluxes in salivary and lacrimal gland acini, and this was ascribed to the then-named P2Z receptor – most likely corresponding to the $P2X_7$ receptor. Now it has been shown that $P2X_7$ receptors are expressed in rodent and human parotid acini and ducts (McMillan *et al.* 1993; Li *et al.* 2003; Brown *et al.* 2004), submandibular gland acini and ducts (Pochet *et al.* 2007; Nakamoto *et al.* 2009; Shitara *et al.* 2009), pancreatic ducts (Christoffersen *et al.* 1998; Hede *et al.* 1999; Novak, 2008) and lacrimal glands (Hodges *et al.* 2009). These and other studies also show that 2′(3′)-*O*-(4-benzoyl-benzoyl)-ATP (BzATP), a commonly used $P2X_7$ receptor agonist, increased reversibly and repetitively cation currents, and acidified cells, while its effects on anion transport are unclear (Henriksen & Novak, 2003; Li *et al.* 2005). BzATP can also stimulate protein secretion (Hodges *et al.* 2009) in exocrine cells. Thus, on one hand, it would seem reasonable to presume that the $P2X_7$ receptors may participate in physiological regulation of epithelial transport. On the other hand, similar to immunoreactive cells, also in exocrine cells, such as salivary acinar cells, excess stimulation of $P2X_7$ receptors can lead to formation of lytic pores, depolarization of mitochondrial membrane, production of reactive oxygen species and apoptosis (Gibbons *et al.* 2001; Garcia-Marcos *et al.* 2005; Seil *et al.* 2008).

Altogether, there is a lot of information about various $P2X_7$ receptor effects on a cellular level, but except for two recent studies (Pochet *et al.* 2007; Nakamoto *et al.* 2009), we do not know about physiological effects of the receptor in a more integrated setting. Therefore, we decided to re-address the physiological function of this receptor in exocrine glands and for this purpose we monitored secretion of major exocrine glands in Pfizer-derived $P2X_7^{-/-}$ mice and studied some cellular events in isolated gland cells. In order to establish the secretory phenotype of the $P2X_7$ receptor, it was also relevant to address the question of whether there is an agonist-induced ATP release in exocrine cells.

Here, we show that several agonists are able to release ATP from pancreas, parotid and lacrimal glands. In *in vivo* experiments on mice we show that cholinergic stimulation elicits secretion in these glands, and notably that $P2X_7^{-/-}$ animals show another secretory phenotype – in pancreas and salivary glands they up-regulate secretion, while in the lacrimal gland they down-regulate secretion. Thus, we propose that $P2X_7$ receptors are involved in physiological regulation of exocrine secretion.

Methods

Materials

All standard chemicals including collagenase, hormones and agonists were obtained from Sigma (Copenhagen, Denmark). Tissue culture media and phosphate-buffered saline (PBS) were from Gibco/Invitrogen (Denmark). Luciferase and luciferin were from Roche Diagnostics (Germany). Mebumal was from Nycomed (Roskilde, Denmark).

Ethical approval

The permission for animal experiments, including the below described protocols, and breeding of transgenic mice was obtained from the Danish Animal Experiment Inspectorate (*Dyreforsøgstilsygnet*). The animal experiments also comply with the guidelines adopted by *The Journal of Physiology* (Drummond, 2009). In order to minimize the number of animals, *in vivo* and *in vitro* functions of the three glands were studied simultaneously (see below).

In vivo collection of exocrine secretion

$P2X_7^{-/-}$ mice on C57BL/6 background were originally obtained from Pfizer (Groton, CT, USA) and wild-type mice of the same strain were obtained from Taconic (Ejby, Denmark) and used for breeding. Mice were housed in standard animal house and had access to chow and water *ad libidum*. We used age- and sex-matched wild-type and receptor knockout animals. They were 20–40 weeks old and weighed 25–40 g. The total number of animals used for this part of the study was 19. For *in vivo* experiments mice were anaesthetized with Mebumal (pentobarbital, 5 mg per 100 g body weight I.P.) and anaesthesia was maintained during the experiments by additional injections of Mebumal. Mice were placed on a heated surgical table, the rectal temperature was monitored and animal was maintained at 38°C. A tracheal cannula was inserted to avoid aspiration of mucus and

saliva. The abdomen was opened by a midline incision and the pylorus and the proximal end of the bile duct were ligated. The common pancreatic bile duct was cannulated with polyethylene tube and pancreatic juice was collected into pre-weighed vials at timed intervals before and after stimulation. Mixed saliva was collected from the mouth at timed intervals. Tears were collected into a polyethylene tube placed at the corner of the eyes and secretion rate was calculated from the volume of fluid collected. Secretion in all glands was evoked by pilocarpine (1 mg (100 g)$^{-1}$, I.P.). At the end of the experiment, animals were killed by an overdose of Mebumal and exocrine glands (pancreas, submandibular, parotid, sublingual and lacrimal glands) and other vital organs were removed, weighed and examined. Secretion rates were corrected per gram of gland weight. Corrections for the body weight gave similar results.

Preparation of cell suspensions

For *in vitro* measurements of ATP release and intracellular Ca^{2+} activities, cell suspensions of three major glands were prepare simultaneously from a single mouse. Mice were killed with cervical dislocation, and pancreas, parotid and exorbital lacrimal glands were removed, placed into cold PBS supplemented with 0.25 mg ml^{-1} trypsin inhibitor and cut into small pieces ($<$1 mm^3). In the lab, solutions were replaced with incubation medium based on Ham's F12/Dulbecco's modified Eagle's medium (DMEM)-1000 mix: 5 ml for pancreas, 3 ml for parotid glands and 2 ml for lacrimal glands. In addition, media contained 1.33 mg ml^{-1} collagenase (type V) (pancreas) or 2.66 mg ml^{-1} (parotid and lacrimal); 0.1 mg ml^{-1} hexokinase or 3 U ml^{-1} apyrase, 0.165 mg ml^{-1} trypsin inhibitor; 0.16 mg ml^{-1} hyaluronidase (lacrimal and parotid preparations) and 1 mM EGTA. Tissues were incubated in rotating bath at 37°C and 5% CO_2 in O_2. At 10 min intervals tissues or cells were dispersed by pipetting and incubation was terminated at 20 min (pancreas) or 30 min (parotid and lacrimal glands) by addition of a cold medium. Cell suspensions were washed and passed through a nylon filter according to procedures described earlier (Hede *et al.* 1999). With this method it was possible to prepare intact acini/small cell clusters and small ducts. For further experiments cells were gently washed in physiological HCO_3^--free buffer (–BIC) of the following composition (in mmol l^{-1}): Na^+ 145, K^+ 3.6, Ca^{2+} 1.5, Mg^{2+} 1, Cl^- 145, phosphate 2.0, glucose 5 and Hepes 10.

ATP measurements

Cells suspended in –BIC solution were pipetted in 50 μl aliquots into 96-well microtitre plates followed by 50 μl of luciferin–luciferase mix from assay kit HSII (Roche Diagnostics, Manheim, Germany) or FL-AA (Sigma), which were dissolved in –BIC. Cells were allowed to rest for 45–60 min. In order to prevent nucleotide hydrolysis (Yegutkin *et al.* 2006), the following nucleotidase inhibitors were also added to each well: 0.3 mM β,γ-methylene-ATP and 2 mM levamizol. Luminescence was monitored directly in cell wells in FLUOStar optima (BMG Labtech, Offenburg, Germany) at 25°C. Luminescence was monitored in 1 s intervals prior to and after injection of 5 μl volumes of –BIC (control) and then an agonist. ATP standards were treated as samples and standard curves were constructed for each experiment. ATP release in response to given agonist was calculated from mean peak responses (10 s) corrected for the control –BIC response due to the mechanical disturbance by the pump injection. ATP release, monitored in arbitrary luminescence units, was recalculated as ATP concentrations. Cells were lysed (according to kit protocols) and assuming that all ATP released originates from intracellular ATP of about 3 mM, one can calculate the number of viable cells. Cell numbers obtained by this method were verified by haematocytometer count in duplicate samples. Concentrations of ATP released from cells following agonist stimulation were corrected for 1 million cells for each sample. The following agonists were used: carbachol (50 μM), cholecystokinin octapeptide (CCK-8; 7×10^{-10} M), neurotensin (1.25×10^{-7} M) and pilocarpine (0.4 mM).

Fura-2 measurements

Cells suspended in –BIC were also used to measure changes in intracellular Ca^{2+} activities using Fura-2. It is well accepted that in exocrine gland cells, agonists increase intracellular Ca^{2+} activity (due to release of Ca^{2+} from intracellular stores and Ca^{2+} influx), and these lead to secretory events, such as stimulation of Ca^{2+} sensitive Cl^- and K^+ channels, and eventually ion and fluid secretion. For simplicity, in the following text we shall refer to changes in intracellular Ca^{2+} activity as Ca^{2+} signals. Fura-2 measurements were made in the microtitre plate reader FLUOStar Optima. In order to minimize movement of cells during pump injections, cells were held on cut-out small filters (Poretics, Sterlitech, Kent, WA, USA) attached with medical adhesive (Hollister, Dansac & Hollister, Fredensborg, Denmark) to the bottom of wells. Cells were loaded with 5 μM Fura-2/AM (Invitrogen) for 30–45 min, then washed and re-suspended in 200 μl of –BIC solution in wells. After further equilibration, Fura-2 signal emission of cells in 3 mm $\times$ 3 mm matrixes was measured at 510–520 nm after excitation at 340 and 380 nm. Cells were stimulated by injection of 5 μl of agonist. The following agonists were used: carbachol (50 μM) and ATP or ATPγS (0.1 mM). The peak change in Fura ratio with a given agonist was taken as a measure of intracellular Ca^{2+} signals.

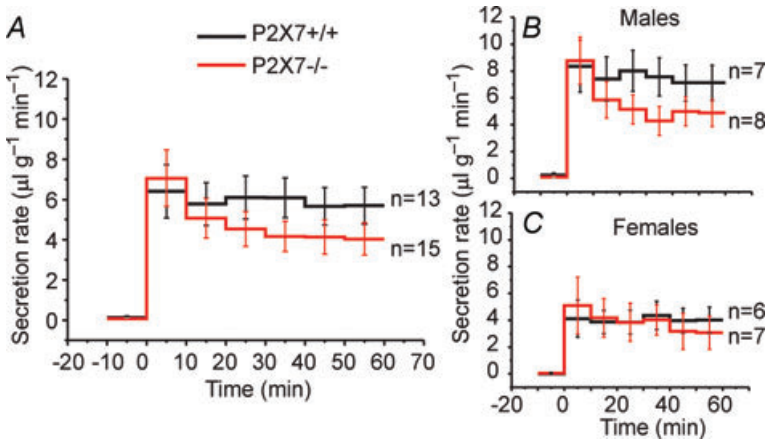

Figure 1. Effect of $P2X_7$ receptor on pancreatic secretion stimulated with cholinergic agonist pilocarpine
Secretory rates are corrected per gland weight and shown for all mice (*A*), males (*B*) and females (*C*). Data points are means ± S.E.M. for *n* experiments.

Microscopy

Cell suspensions of exocrine glands were examined with a confocal laser scanning microscope (Leica SP CLSM equipped with an Ar–Kr laser) with 20× 1.7 NA HC PL APO and 63× 1.2 NA PL APO objectives. In order to estimate ATP stores, cells were incubated with 1–5 μM quinacrine dihydrochloride for 5–15 min and fluorescence was detected at 490–540 nm with 476 nm excitation (Sørensen & Novak, 2001). Exocrine glands were also fixed in 4% parafomaldedyhe and stained with haematoxylin/eosin and general morphological structure was examined by light microscopy.

Data presentation

Data are presented as original recordings and summaries showing the mean values ± S.E.M. with *n* indicating a number of independent experiments. For analysis of responses ANOVA or Student's *t* test was used. Data were analysed in Origin (OriginLab Corp., Northampton, MA, USA).

Results

Pancreatic, salivary and lacrimal gland secretions

$P2X_7$ receptors have various effects on exocrine glands at a cellular and subcellular level. In order to test whether these receptors also affect exocrine secretion, we set up *in vivo* experiments on $P2X_7^{+/+}$ and $P2X_7^{-/-}$ mice that were stimulated with pilocarpine, a muscarinic receptor agonist that is a good secretagogue of epithelial secretion. Simultaneously, we collected pancreatic secretion, saliva and tears prior to and after stimulation and the results are shown in Figs 1–3. Secretory rates were corrected for the gland weight, as at the end of the experiments pancreas, major salivary glands (submandibular, parotid and sublingual) and exorbital lacrimal glands were dissected and weighed. Similar results as depicted in Figs 1–3 were obtained when secretory rates were corrected for animal weight.

Figure 1 shows secretion rates (per gram of gland) of pancreatic juice of $P2X_7^{+/+}$ and $P2X_7^{-/-}$ mice as a function of time. Non-stimulated secretion was relatively low, and pilocarpine evoked relatively high secretion rates

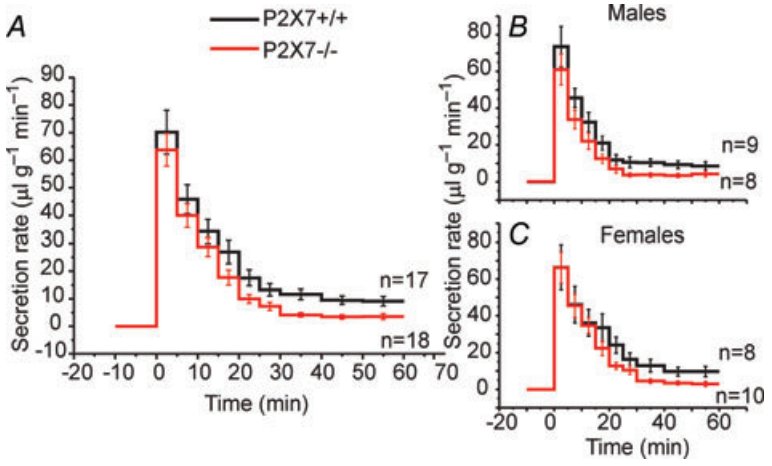

Figure 2. Effect of $P2X_7$ receptor on mixed salivary secretion stimulated with pilocarpine
See Fig. 1 legend for details.

in both wild-type and receptor knockout animals. In a relatively large number of animals of both sexes, it became apparent that after the initial secretory response (10–20 min), pancreas of $P2X_7^{-/-}$ animals appeared to secrete at about 30% lower rate (Fig. 1*A*). In order to determine whether there were any sex differences, we analysed the data as shown in Fig. 1*B* and *C*. Clearly, the female pancreas of all animals secreted at lower rates than the male pancreas, and there seemed to be no differences between the wild-type and knockout mice. Integrated 60 min secretion was $242 \pm 51\ \mu l\ g^{-1}\ h^{-1}$ in wild-type and $234 \pm 82\ \mu l\ g^{-1}\ h^{-1}$ in knockout female mice ($n = 6$ and 7). Wild-type males secreted significantly more pancreatic juice (per gland or body weight), and knockout of the receptor appeared to decrease secretion. Integrated 60 min secretion was $438 \pm 69\ \mu l\ g^{-1}\ h^{-1}$ in wild-type and $339 \pm 72\ \mu l\ g^{-1}\ h^{-1}$ in knockout male mice ($n = 7$ and 8). This effect was more pronounced after prolonged stimulation, i.e. mean secretion at 20–60 min was $7.46 \pm 0.21\ \mu l\ g^{-1}\ min^{-1}$ in wild-type and $4.82 \pm 0.18\ \mu l\ g^{-1}\ min^{-1}$ in $P2X_7^{-/-}$ male mice ($n = 7, 8$), which was 35% lower ($P = 8.8 \times 10^{-5}$).

Compared to pancreas, 60 min salivary secretion was much higher and amounted to $1341 \pm 154\ \mu l\ g^{-1}\ h^{-1}$ in all wild-type mice and $931 \pm 91\ \mu l\ g^{-1}\ h^{-1}$ in $P2X_7^{-/-}$ mice ($n = 17$ and 19; $P = 0.024$). Figure 2 shows salivary secretion rates as a function of time; the typical biphasic response may be due to different sets of ion transporters contributing to net secretion with prolonged stimulation (Novak & Young, 1986). Again in order to detect possible sex differences, data are plotted for male and female mice in Fig. 2*B* and *C*. In the peak phase of secretion (arbitrarily defined integrated 0–30 min of secretion), $P2X_7^{+/+}$ male mice secreted significantly higher than $P2X_7^{-/-}$ mice, i.e. 990 ± 110 *vs.* $626 \pm 74\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ ($P = 0.0237$). In the plateau phase (30–60 min), the total secretion was $267 \pm 63\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ *vs.* $114 \pm 39\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ in the same animals ($n = 9$ and 8). In female animals, the initial integrated peak secretion (0–30 min) was similar, i.e. $1114 \pm 205\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ in wild-type and $960 \pm 110\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ in receptor knockout animals. In the same animals, the plateau integrated secretion (30–60 min) was $323 \pm 85\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ in $P2X_7^{+/+}$ mice and significantly lower $109 \pm 31\ \mu l\ g^{-1}\ 0.5\ h^{-1}$ in $P2X_7^{-/-}$ animals ($n = 8$ and 10; $P = 0.021$). Taken together, the secretory patterns were similar in salivary glands of both male and female mice (Fig. 2*B* and *C*). However, in the peak phase of secretion, male glands appeared to be more affected by the receptor knockout, while in the plateau phase of secretion (30–60 min), all knockout animals had secretion of about half that observed in wild-type animals, i.e. mean secretion at 30–60 min was $9.94 \pm 1.68\ \mu l\ g^{-1}\ min^{-1}$ in wild-type and $3.81 \pm 0.79\ \mu l\ g^{-1}\ min^{-1}$ in $P2X_7^{-/-}$ mice ($n = 17$ and 18), which was 62% lower ($P = 0.019$).

Tears are produced by several glands/epithelia, and the most significant contribution is from the lacrimal glands. Figure 3*A* shows that pilocarpine stimulation increased tear production 3.8-fold in wild-type animals, i.e. from 2.07 ± 0.88 to $7.95 \pm 1.35\ \mu l\ g^{-1}\ min^{-1}$ ($n = 16$; $P = 0.002$), and 4.3-fold in knockout animals, i.e. 2.49 ± 0.81 to $10.76 \pm 1.63\ \mu l\ g^{-1}\ min^{-1}$ ($n = 7$; $P = 7.2 \times 10^{-5}$). There seemed to be larger tear production in $P2X_7^{-/-}$ animals, and so we re-analysed the data taking sex into account (Fig. 3*B* and *C*). Data show that wild-type males and females had similar resting tear secretion, i.e. $2.98 \pm 1.47\ \mu l\ g^{-1}\ min^{-1}$ and $0.90 \pm 0.53\ \mu l\ g^{-1}\ min^{-1}$ ($n = 9$ and 7; $P = 0.252$). The cholinergic stimulation increased secretion in these animals to $8.79 \pm 1.69\ \mu l\ g^{-1}\ min^{-1}$ in males and $6.88 \pm 2.89\ \mu l\ g^{-1}\ min^{-1}$ in females. In $P2X_7^{-/-}$ animals secretion was significantly higher in males compared to females in the resting state, i.e. $4.41 \pm 1.42\ \mu l\ g^{-1}\ min^{-1}$ compared to $0.76 \pm 0.40\ \mu l\ g^{-1}\ min^{-1}$, respectively ($P = 0.019$); and in the stimulated state it was $15.81 \pm 2.07\ \mu l\ g^{-1}\ min^{-1}$

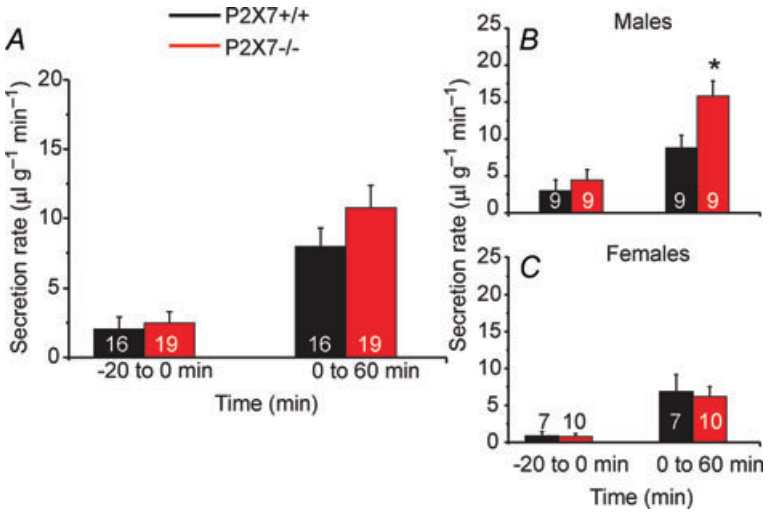

Figure 3. Effect of $P2X_7$ receptors on tear secretion stimulated with pilocarpine
Tear secretion was collected 20 min before stimulation and 60 min following stimulation and secretory rates corrected for gland weight were calculated. $P2X_7^{-/-}$ males have significantly higher secretion compared to $P2X_7^{+/+}$ males ($P = 0.018$). $P2X_7^{-/-}$ males have higher tear secretion in non-stimulated and also stimulated state compared to females ($P = 0.019$; $P = 0.001$).

for males and $6.21 \pm 1.35\ \mu l\ g^{-1}\ min^{-1}$ for females ($n = 9$ and 10; $P = 0.001$). Notably, as Fig. 3*B* shows, the $P2X_7$ receptor knockout in males increased tear secretion by 80% ($P = 0.018$). For comparison with other glands, the 60 min integrated secretion was $527 \pm 237\ \mu l\ g^{-1}\ h^{-1}$ and $413 \pm 137\ \mu l\ g^{-1}\ h^{-1}$ in wild-type males and females; and $924 \pm 124\ \mu l\ g^{-1}\ h^{-1}$ and $373 \pm 81\ \mu l\ g^{-1}\ h^{-1}$ in knockout males and females respectively. Note that these apparent high secretory rates are due to the fact that they were corrected for gland weights, i.e. 0.02–0.03 g per two lacrimal glands.

ATP release

The above data show that the $P2X_7$ receptor seems to be important for fluid secretion in all three types of glands, thus implying that the ligand, ATP, is released within the glands. Our working hypothesis is that cholinergic stimulation of exocrine glands results in release of ATP, which via interaction with $P2X_7$ receptors leads to co-regulation of exocrine secretion. Firstly, we needed to test that cholinergic stimulation can release ATP in the glands involved, as this has only been shown convincingly for pancreas (Sørensen & Novak, 2001; Kordas *et al.* 2004; Yegutkin *et al.* 2006). Secondly, we needed to test that the $P2X_7$ receptor expression did not have an effect on ATP stores indirectly or directly and in the latter case if it contributes to ATP release as suggested by some studies (Pellegatti *et al.* 2005). To address these questions, we prepared cell suspensions (containing acini and ducts) from three major exocrine glands of interest – pancreas, parotid gland and lacrimal gland. In one series of experiments, we loaded freshly prepared cells with quinacrine, which is an indicator of ATP stores (Sørensen & Novak, 2001). Figure 4 shows representative fluorescence and transmission images from 6–10 scans made on preparations from all three types of glands obtained from $P2X_7^{+/+}$ and $P2X_7^{-/-}$ animals. Notably, quinacrine was accumulated in granules/vesicles in acini in all gland types. There seems to be no difference between glands obtained from wild-type or receptor knockout animals. That is, the mean fluorescence intensity (grey levels) was 111 ± 5 in acini from wild-type and 108 ± 8 from acini in knockout animals ($n = 28$ and 7). Another interesting observation was that as in the pancreatic ducts (Sørensen & Novak, 2001), parotid and lacrimal gland ducts showed weaker quinacrine fluorescence in similar regions of interest, and quinacrine was localized in small vesicles as shown in Fig. 4*B*. That is, the quinacrine intensity in ducts was 46 ± 4 ($n = 28$), which was significantly lower than in acini ($P < 10^{-7}$); and vesicle size was $0.92 \pm 0.05\ \mu m$ in ducts compared to $1.10 \pm 0.03\ \mu m$ in acini ($n = 13$ and 31; $P = 0.003$). As shown in the earlier study on pancreatic acini (Sørensen & Novak, 2001), carbachol stimulation resulted in a decrease in the quinacrine fluorescence in luminal regions of acinar cells, indicating that quinacrine/ATP has been released.

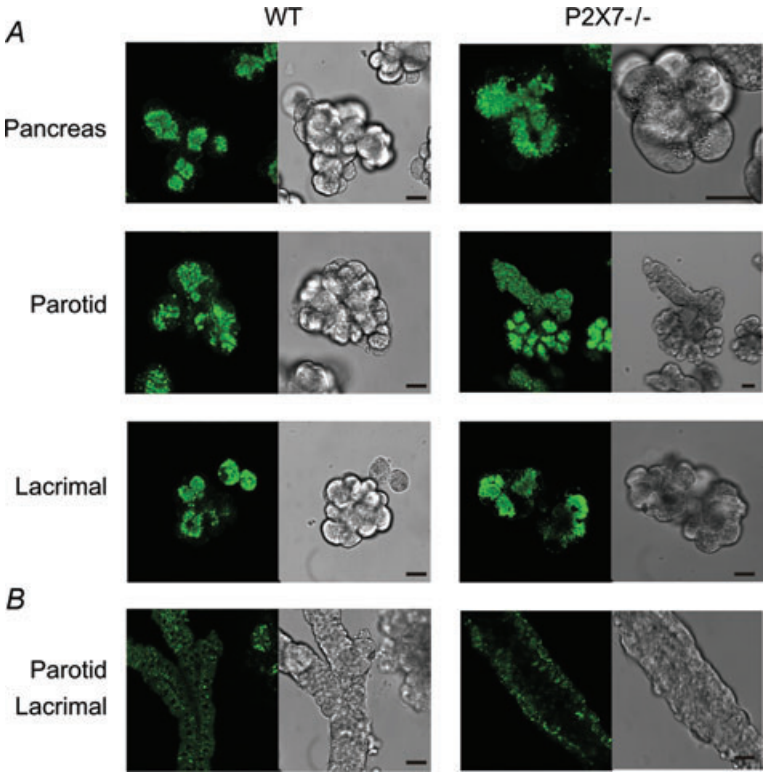

Figure 4. ATP stores in exocrine glands are not affected by $P2X_7$ receptor expression
A, representative images of quinacrine labelled acini (green) and corresponding transmission images. *B*, ducts from the parotid and lacrimal glands in $P2X_7^{+/+}$ and $P2X_7^{-/-}$ mice as indicated. Note also parotid duct in *A*. All bars are 20 μm.

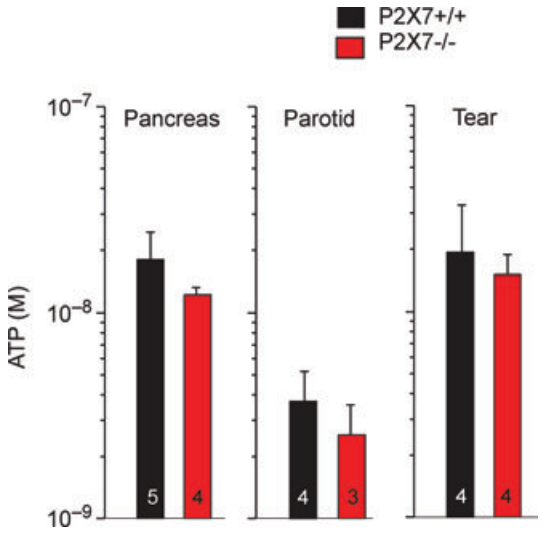

Figure 5. Effect of cholinergic stimulation on ATP release
Exocrine cells prepared from pancreas, parotid and lacrimal glands of P2X$_7^{+/+}$ and P2X$_7^{-/-}$ mice. Bars show means ± S.E.M. and number of experiments.

In the next series of experiments we set out to determine the ATP release directly using the luciferin/luciferase method. Since we have earlier established that pancreatic cells express ecto-nucleotidases (Yegutkin *et al.* 2006), nucleotidase inhibitors were included in the cell suspension. First we focused on the effect of cholinergic stimulation on ATP release in wild-type and knockout animals. Figure 5 depicts the results, which show that there was no difference in ATP release in the two groups of animals. This supports the data with the putative ATP-store marker quinacrine (Fig. 4). We also show that other agonists can also elicit ATP release (Fig. 6). Pilocarpine, given at concentrations that we estimated would be the case following I.P. administration in *in vivo* experiments, caused release of ATP from the three types of glands. Neurotensin had a similar effect in all three glands. CCK-8 also released ATP in pancreas, as established earlier also in *in vivo* preparations (Yegutkin *et al.* 2006).

Following ATP release in glands, ATP would activate various P2 receptors, including P2X$_7$ receptor, if present. Since most G-protein coupled P2Y receptors and cation permeable P2X receptors lead to intracellular Ca^{2+} signals, we measured the change in Fura-2 ratio in cell suspensions from our gland preparations in response to ATP or ATPγS, which is less prone to hydrolysis, and Fig. 7*A* shows the results. In parotid and lacrimal glands from P2X$_7^{+/+}$ and P2X$_7^{-/-}$ animals, ATP induced similar Ca^{2+} signals. Notably, in pancreatic cells from P2X7$^{-/-}$ animals, Ca^{2+} signals were significantly lower, i.e. the ΔFura-2 ratio decreased from 0.076 to 0.036 ($n = 14$ and 16; $P = 0.030$). This indicates that indeed the contribution from the P2X$_7$ receptor was lost in pancreas. In addition, we also tested the effect of muscarinic agonist on these gland preparations and the results are shown in Fig. 7*B*. The simplest assumption is that carbachol stimulation of muscarinic receptors would have led to Ca^{2+} signals due to Ca^{2+} store release and Ca^{2+} influx. Alternatively, or in addition, carbachol could have induced ATP release and subsequent Ca^{2+} signals in the same or other cells in the preparation. The results show that carbachol had similar effects on Ca^{2+} signals in pancreatic cells. However, in both parotid and lacrimal cells, carbachol increased Ca^{2+} signals by 70% and 63% in preparations from P2X$_7^{-/-}$ animals compared to wild-type animals.

Since there appeared to be some sex differences in exocrine secretion from the three types of glands (Figs 1–3), we examined possible differences in intracellular Ca^{2+} activities. We could not detect any significant difference in pancreatic and parotid cells (data not shown); however, there was a clear difference in lacrimal cells (Fig. 8). Carbachol-induced Ca^{2+} signals were similar in females from P2X$_7^{+/+}$ and P2X$_7^{-/-}$ glands, which were

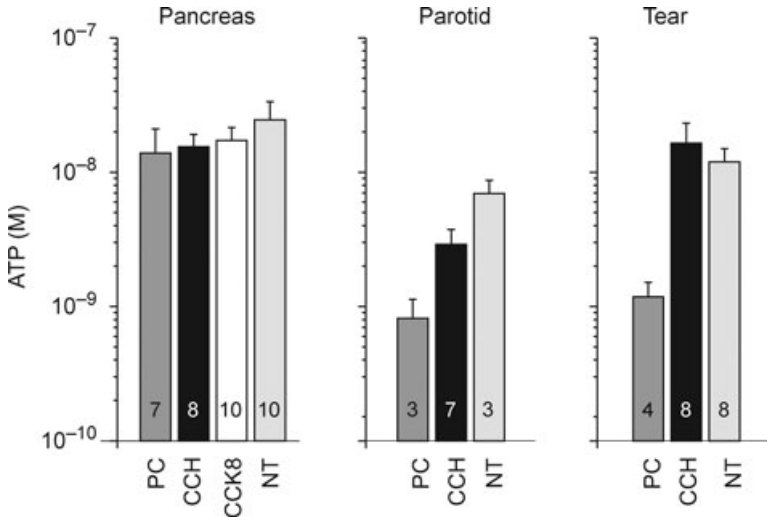

Figure 6. Effect of various agonists on ATP released from exocrine cells prepared from various glands
Agonists used were pilocarpine (PC, 0.40 mM), carbachol (CCH, 50 μM), neurotensin (NT, 0.12 μM) and cholecytokinin octapeptide (CCK-8, 0.7 nM). Bars show means ± S.E.M. of ATP concentrations, and *n* is the number of experiments, where glands from P2X$_7^{+/+}$ and P2X$_7^{-/-}$ animals were pooled.

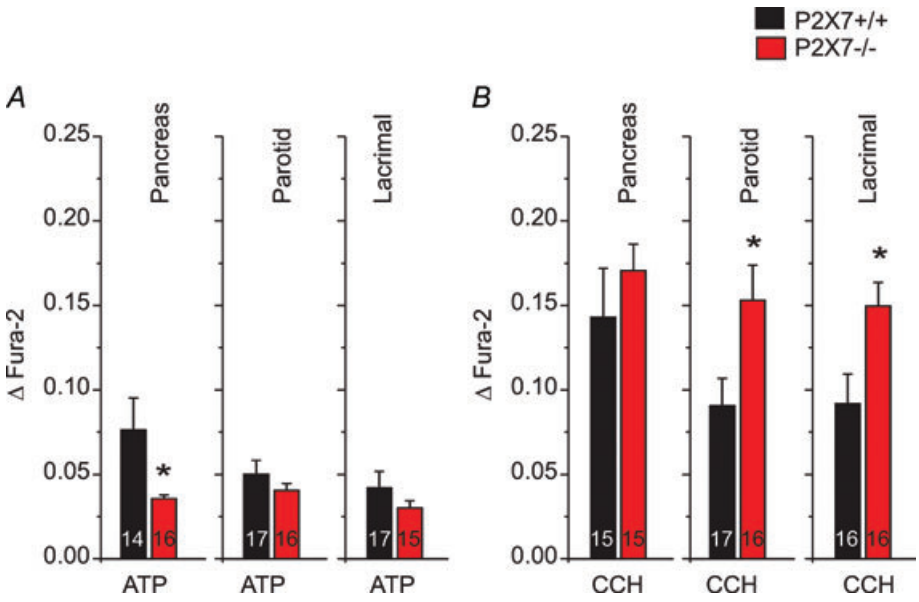

Figure 7. Effect of the ATP analogue ATPγS (*A*) and cholinergic stimulation (*B*) on intracellular Ca^{2+} signals in various glands
ΔFura-2 denotes change in the Fura-2 ratio with agonist stimulation. *A*, ATPγS (0.1 mM) induced significantly lower Ca^{2+} signals in $P2X_7^{-/-}$ pancreas compared to wild-type pancreas ($P = 0.030$). *B*, carbachol (50 μM) induced higher Ca^{2+} signals in both parotid and lacrimal glands of $P2X_7^{-/-}$ animals compared to glands from wild-type mice ($P = 0.024$ and $P = 0.015$, respectively).

paralleled by similar secretion rates (Fig. 3). However, in cells from $P2X_7^{-/-}$ male mice, Ca^{2+} signals were about three times higher than in animals with functional $P2X_7$ receptors (i.e. ΔFura-2 increased from 0.059 ± 0.012 to 0.169 ± 0.023, $n = 7$ and 8; $P = 0.015$), and this effect was also reflected in higher tear secretion in these animals (Fig. 3).

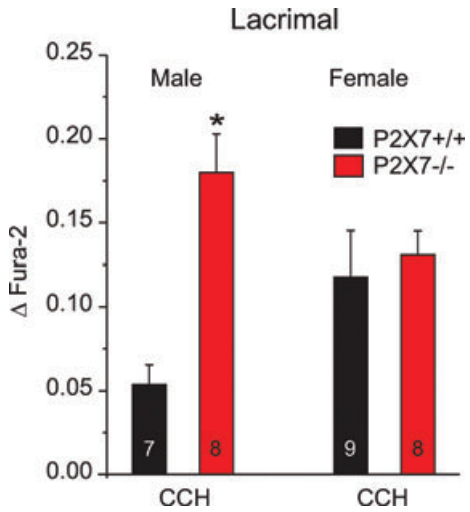

Figure 8. Sex differences and $P2X_7$ receptors in lacrimal glands
Ca^{2+} signals (ΔFura-2) were monitored in lacrimal gland cells stimulated with carbachol (50 μM). Cell suspensions were prepared from male and female wild-type and $P2X_7^{-/-}$ mice. In male glands receptor knockout resulted in significantly higher Ca^{2+} signals.

Discussion

The most important finding in the present study is that the $P2X_7$ receptor knockout affects secretion of three types of exocrine glands. We cannot exclude that expression of other P2Y and P2X receptors or transport proteins involved in secretion was also affected. However, we argue for the simplest interpretation, namely that the $P2X_7$ receptors are physiologically relevant in epithelial secretion. They can up-regulate or down-regulate secretion, depending on the gland type, origin of the gland (male or female), and interaction with cholinergic signalling within the specific gland. Based on our study, we propose that the physiological sequence of events in exocrine glands is cholinergic stimulation, ATP release, stimulation of $P2X_7$ receptors and possible interaction with muscarinic receptors, intracellular Ca^{2+} signals, and finally the fluid secretion. These issues are taken up in the following discussion.

ATP release

The fact that pilocarpine stimulation has different effects on secretion in glands from $P2X_7^{+/+}$ and $P2X_7^{-/-}$ animals (Figs 1–3) indicates that $P2X_7$ receptors are stimulated by ATP released within the glands following cholinergic stimulation. This is confirmed by our experiments on gland cell suspensions, where we show that carbachol and pilocarpine (and other agonists) release ATP from

isolated exocrine cells (Figs 5 and 6). Therefore, the theory first proposed and validated for pancreas (Sørensen & Novak, 2001; Novak, 2003; Yegutkin *et al.* 2006) is also tangible for salivary and tear glands. That is, exocrine cells release/secrete ATP in response to physiological stimuli and this ATP affects the same cells in an autocrine manner or the neighbouring cells in a paracrine manner (e.g. downstream cells such as duct cells in an intact gland).

ATP release mechanisms are currently an issue of intense research and several candidates involved in non-vesicular and vesicular release have been proposed, including connexins, voltage-dependent anion channels, CFTR and even P2X$_7$ receptor regulated pannexins (Yegutkin, 2008; Praetorius & Leipziger, 2009). Our study shows that ATP release is similar in glands from both wild-type and P2X$_7$ receptor knockout animals (Fig. 5). This shows that the P2X$_7$ receptors are not involved in regulation of ATP release alone or in combination with pannexin-1, as proposed for other cells by other workers (Pellegatti *et al.* 2005; Pelegrin & Surprenant, 2006; Locovei *et al.* 2007). Quinacrine, an indicator of ATP stores, was distributed in vesicular stores in parotid and lacrimal glands (Fig. 4). This is similar to localization in pancreas described earlier (Sørensen & Novak, 2001), and as we show recently, ATP is accumulated in zymogen granules by vesicular nucleotide transporter VNUT (Haanes & Novak, 2010).

ATP is released into the lumen of glands (Fig. 4*B*) (Sørensen & Novak, 2001; Sørensen *et al.* 2003; Ishibashi *et al.* 2008), as proposed for other epithelia (Schwiebert & Zsembery, 2002; Leipziger, 2003; Novak, 2003), ATP and other nucleotides/sides have been detected in pancreatic juice and also on the eye surface (Sørensen & Novak, 2001; Yegutkin *et al.* 2006; Crooke *et al.* 2008). The amount and composition of nucleotides detected on epithelial surfaces or secretions will also depend on ecto-nucleotidase (and kinase) activities, which are high in exocrine gland cells (Sørensen *et al.* 2003; Yegutkin *et al.* 2006). Furthermore, the purinergic signalling in the natural microenvironments of the organ may include more components and our simplified experiments do not permit us to conclude whether ATP is also released from nerve endings, or whether ATP release also occurs across the basolateral membranes of epithelial cells.

Secretion – gland by gland

The general patterns of secretion of the three gland types observed in this study are similar to published studies on *in vivo* and *in vitro* preparations of glands stimulated by cholinergic or hormonal agonists (Sewell & Young, 1975; Novak & Young, 1986; Walcott *et al.* 2005; Nakamoto *et al.* 2009). Regarding the P2X$_7$ receptor, at first, it may seem surprising that the receptor knockout had a different effect on responses of the three types of exocrine glands. Nevertheless, the glands have different functions, probably a different constellation and regulation of transporters. Purinergic system may be a part of this regulation, and glands can in fact express different P2X$_7$ receptor splice variants (Nicke *et al.* 2009).

In pancreas, the major sites of ATP release are acini, which themselves show low functionality of purinergic receptor signalling and apparent lack of P2X$_7$ receptors (Simeone *et al.* 1995; Novak *et al.* 2002). Various P2 receptors, including P2X$_7$ receptors are expressed and highly functional in rodent and human ducts, where they are associated with Ca^{2+} signals, Na^+/Ca^{2+} flux and exchange, H^+/HCO_3^- transport and ERK stimulation (Henriksen & Novak, 2003; Hansen *et al.* 2008; Novak, 2008). In contrast to other exocrine glands, pancreatic ducts are clearly contributing to significant fluid secretion, which is rich in $NaHCO_3$. Most likely, P2X$_7$ receptors co-regulate ductal secretion evoked by other agonists, e.g. cholinergic stimulation. In the present study on pancreatic cells (acini and ducts), it is clear that ATP elicited smaller Ca^{2+} signals in cells from P2X$_7^{-/-}$ animals (Fig. 7), showing that the receptor contributed to this event, and also to secretion that most likely originates in ducts (Fig. 1).

In salivary and lacrimal glands, the main contributors to fluid secretion are acini and they express functional P2 receptors and intracellular signalling (see Introduction). Salivary secretion measurements in the present study were done on whole saliva contributed by the major and minor salivary glands, though the major volume of secretion is contributed by the submandibular and parotid glands and both express P2X$_7$ receptors (Introduction). As may have been expected then, the P2X$_7$ receptor knockout reduced salivary secretion (Fig. 2). There are two other relevant secretion studies on salivary glands of similar P2X$_7$ knockout animals. Nakamoto *et al.* (2009) studied *ex vivo* perfused sumandibular gland from wild-type and P2X$_7$ knockout animals and found that P2X$_7$ receptor stimulation was responsible for about 70% of fluid secretion, which would support our findings. In contrast, Pochet *et al.* (2007) measured bulk salivary secretion in wild-type and P2X$_7$ knockout mice after stimulation with pilocarpine, but could not detect any difference in the first 20 min of fluid secretion between these animals. This anomaly could be explained by our observation showing that the receptor knockout effect in pilocarpine saliva is most apparent in the plateau phase of secretion (after some 20 min). Interestingly, in this phase of secretory response, the saliva secretion is driven by ion transporters associated with H^+/HCO_3^- transport rather than the Na^+–K^+–Cl^- and Cl^- transporters (Novak & Young, 1986). Thus, we can predict some parallels between pancreatic and salivary gland secretions – in regulation and cellular mechanisms.

The aqueous layer of the tear film contains proteins, electrolytes and water, which are mainly secreted by the

lacrimal gland; minor secretion containing lipids is mainly by the Meibomian glands; and mucus is produced by epithelial and goblet cells (Crooke *et al.* 2008). It is reasonable to assume that tear fluid secretion would be mainly contributed by lacrimal gland cells, which we used in this study. The cellular model for secretion is well studied on isolated acini (and the latest also with ducts) and is similar to salivary and pancreatic acini (Dartt, 2009). A recent paper by Dartt's group (Hodges *et al.* 2009) shows that $P2X_7$ receptors are expressed in lacrimal acini of male rats – where they stimulate Ca^{2+} signals, ERK1/2 and protein secretion. It is perhaps unexpected then that our study shows that $P2X_7$ receptor knockout in mice had no effect on fluid secretion (females) or even increased fluid secretion (males) (Fig. 3). The simplest proposal is that protein and fluid secretions are not regulated in parallel by the $P2X_7$ receptors. Another suggestion is that there are interactions between cholinergic (muscarinic receptor) and purinergic signalling. This interaction may be partly reflected by Ca^{2+} signals in all glands. Although the Ca^{2+} signal measurements are relatively crude in our study, as we monitored global peak changes in cell suspensions containing acini and ducts, we already see some interesting patterns worth discussing below.

Interaction of purinergic and cholinergic system and calcium signals

Interaction between purinergic receptors and other receptors can occur at several levels: at the plasma membrane level, via interaction of intracellular signalling pathways, and at the organ level. On the level of plasma membrane, G-protein coupled receptors can heteromerize and also ligand-gated receptors, such as P2X and nicotinic receptors, exhibit physical cross-talk (Khakh *et al.* 2000; Koles *et al.* 2008). At the organ level, Tenneti *et al.* (1998) have shown that there is trans-synaptic regulation of receptor-mediated signalling between muscarinic and P2X receptors, such that removal of the autonomic innervations increases ATP responses (presumably via $P2X_4$ and $P2X_7$ receptors) in parotid acinar cells. From our study it seems, though, that this is not the other way around, i.e. removal of $P2X_7$ receptors did not lead to better secretion by cholinergic stimulation (at least in the salivary glands and pancreas), although Ca^{2+} signals were increased (in salivary glands).

In pancreas, clearly knockout of the $P2X_7$ receptor led to lower Ca^{2+} signals (in response to ATP) and secretion was decreased. This secretion most likely originated in ducts rather than acini, as the former contribute significantly to secretion and express many functional P2 receptors, while acini do not (Hede *et al.* 1999; Novak *et al.* 2002). In parotid and lacrimal cells, $P2X_7$ receptor knockout had no significant effect on ATP-mediated Ca^{2+} signals in cell preparations, most likely because these acini express many functional P2 receptors that can substitute for $P2X_7$ receptor knockout (see Introduction). Nevertheless, the receptor knockout led to increased Ca^{2+} signals in response to muscarinic receptor stimulation. This is in accordance with findings of Hurley *et al.* (1993) on submandibular gland acini, which showed that $P2X_7$ receptors prevented Ca^{2+} release from intracellular stores by carbachol and also adrenaline. Similar findings were made by Jørgensen *et al.* (1995) on parotid acini, where the $P2Z/P2X_7$ receptor diminished the effect of the muscarinic receptor on release of inositol 1,4,5-trisphosphate and store Ca^{2+}. Therefore, it was expected that $P2X_7$ knockout would improve Ca^{2+} signals (Fig. 7). But receptor knockout did not rescue pilocarpine-induced total salivary gland secretion in intact animals; in fact it decreased it (Fig. 2). Though for the isolated submandibular glands from mice of both sexes, Nakamoto *et al.* (2009) reported that $P2X_7$ receptor knockout increased muscarinic receptor induced fluid secretion. This latter observation is more in accordance with what we propose for the lacrimal glands. That is, in lacrimal glands, $P2X_7$ receptor knockout clearly improved both Ca^{2+} signalling and secretion, particularly in the males (Figs 3 and 8).

Taken together, in some glands the muscarinic and $P2X_7$ receptors interact, most likely via signalling pathway that converge and decrease Ca^{2+} signals, which eventually increases secretion in salivary glands but decreases it in lacrimal glands. It is then important to consider that increased Ca^{2+} signals may not be synonymous with increased secretion. This anomaly is also seen with $P2Y_2$ receptor stimulation, which leads to large Ca^{2+} signals, yet inhibits specific K^+ channels (BK) and secretion (Hede *et al.* 1999). Thus, it will be necessary to look beyond global Ca^{2+} signals (detected in this study) to specific exocrine cells (acini and ducts), specific ion transporters and localized subcellular signals and involvement of other signalling macromolecules, such as mitogen activated protein kinases and members of the PKC family that can up- and down-regulate plasma membrane transporters (Rosse *et al.* 2010).

Sex differences

In this study we detected sex differences in exocrine secretion (Figs 1 and 3). Sex hormones affect development, structure and function of many organs, including epithelia (Sabolic *et al.* 2007). Regarding morphology, there is well documented sexual dimorphism in rodent salivary glands, where male glands contain well developed granular convoluted tubules containing granules with growth factors and kallikrein (Barka, 1980; Jayasinghe *et al.* 1990; Tandler *et al.* 2001). Male glands also contain smaller volume of acini, which may explain their lower secretion (Fig. 2). Regarding sexual dimorphism and

function, some of the hormonal effects include rapid non-genomic responses by influencing intracellular Ca^{2+}, PLC and various kinases (PKC, PKA, MAPK) and ion channels. Relevant to our study may be the finding that 17β-oestradiol inhibits Cl^- secretion in colonic crypts, and this seems to be exerted via inhibition of a K^+ channel, KCNQ1, that is regulated via PKCδ and PKA activation (O'Mahony *et al.* 2007). Similar mechanism could explain lower secretion in female pancreas and tear glands compared to male glands.

Most interesting is the observation that we also detect difference in the effect of $P2X_7$ receptor knockout in male and female glands. Most importantly, the $P2X_7$ receptor knockout had no effect on secretion in all female exocrine glands. In males, though, the receptor knockout decreased secretion in pancreas (and salivary glands) but increased it in lacrimal glands. These effects do not seem to be related to ATP release (Fig. 5), but may be directly related to the $P2X_7$ receptor, such that in females, for example, oestradiol would inhibit the $P2X_7$ receptor. There are a few studies that support our observations regarding the issue of $P2X_7$ receptor and sex differences and/or oestradiol. In studies on bone remodelling in mice, the $P2X_7$ receptor knockout had less obvious effects on the loss of bone mass of females compared to males (Ke *et al.* 2003). In post-menopausal women oestrogen treatment prevented loss of bone mass (Ohlendorff *et al.* 2007). On the cellular level, two studies indicate that the $P2X_7$ receptor function is partly regulated by oestrogens. In studies on monkey kidney COS cells expressing the $hP2X_7$ receptor, but not the oestrogen receptor, it was shown that 17β-oestradiol had rapid inhibitory effects on cation flux through the receptor (Cario-Toumaniantz *et al.* 1998). A similar inhibitory effect of oestrogen on Ca^{2+} influx, and also on apoptosis associated with the pore formation, was observed in uterine cervical cells (Gorodeski, 2004).

In conclusion, our study shows that the $P2X_7$ receptors have effects on secretion of three major exocrine glands and we propose that they play a role in physiological regulation. We show that the signalling molecule, ATP, can be released from glands following cholinergic stimulation and this is independent of $P2X_7$ receptors. Furthermore, we show that $P2X_7$ receptors act in conjunction with muscarinic receptors to increase secretion (pancreas, salivary glands) and to decrease secretion (tear glands) and these latter effects are related to male/female origin of the gland.

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Author contributions

I.N. conceived and designed the study, performed *in vivo* secretion experiments and confocal microscopy and wrote the paper. I.M.J. and L.W. performed *in vitro* experiments on cell suspension and contributed to data analysis and interpretation. All authors approved the final version of the paper.

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